Polymer Nanocomposite Films Based on Halloysite Modification for Potential Biomedical Applications

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INTRODUCTION

Biocompatible materials have been integral to biomedical fields for decades. These materials can be modified on the surface to regulate their properties, influencing cellular activities in specific environments like the extracellular matrix (ECM). Both natural and synthetic biomaterials have unique advantages, such as supporting cell growth and controlled biodegradation. They are extensively studied for applications like wound healing, bone regeneration, and tissue engineering, requiring an understanding of cellular behaviour such as cell adhesion, proliferation, and cytocompatibility [1]. Polymer nanocomposites, which incorporate nanoscale particles into a polymer matrix, are a growing area of research due to their enhanced properties like improved strength and catalytic activity. Nanocomposites are categorized by their matrix materials (ceramic, metallic, or polymer), and incorporating natural nanomaterials into polymer matrices is gaining traction due to their eco-friendly nature [2].

These biomaterials are extensively researched for wound healing, bone regeneration and tissue engineering purposes, necessitating the study of cellular behaviour aspects such as cell proliferation, adhesion, and cytocompatibility properties [3]. By blending and shaping materials into porous structures, films, and scaffolds with customized architectures, their thermo-mechanical properties can be effectively adjusted. Naturally occurring biopolymers such as chitosan, pectin, sodium alginate, silk, cellulose, and collagen are widely utilized in scaffold fabrication for wound healing and tissue engineering. Combining these natural biopolymers with synthetic counterparts allows for the creation of materials that mimic biological structures, enabling precise targeting for biological research and related applications [4].

In an era marked by the expanding utilization of scientific knowledge, polymer nanocomposites based biomaterials are emerging as a focal point in numerous research and development endeavours. Polymer nanocomposites refer to materials wherein nanoscale inorganic particles, typically measuring 10-100A° in at least one dimension, are dispersed within an organic polymer matrix [5]. Nano-sized particles have witnessed a recent surge in their applications owing to their diminutive size, heightened catalytic activities, and expansive surface areas. Through the straightforward addition of nanoparticles to a polymer matrix, its performance can be significantly augmented, utilizing the inherent nature and properties of the nanoscale filler.

Nanocomposites are classified into three types based on their matrix materials: ceramic, metallic, and polymer matrix [6]. Extensive efforts are underway to prepare polymer composites by incorporating naturally occurring

nanomaterials as fillers. The combination of high-strength fibres such as carbon, glass, and aramid with a low-strength polymer matrix now dominates various industries including aerospace, automotive, construction, and sports. However, the use of these fibres has led to several drawbacks such as non-biodegradability, non-recyclability, and high energy consumption. Therefore, there is a growing interest in synthesizing biocompatible polymer nanocomposites using naturally or synthetically available nanofillers. Our research focuses on selecting Halloysite nanotubes (HNT) as a reinforcement material for the polymer matrix.

Halloysite, discovered in the 18th century, was named in honor of O. d'Halloy who analysed the mineral [7]. HNT is a dioctahedral aluminosilicate 1:1 clay mineral with the chemical formula $Al_2(OH)_4Si_2O_5.nH_2O$, existing in both hydrated and dehydrated forms. Over time, its structural understanding has progressed from being considered a solid tube-like structure to being recognized as a series of layered, folded formations with porous walls composed of aluminium oxide and silicon oxide [8]. Water molecules are intercalated between these layered structures. The hydrated form occurs when n = 2, with water molecules present between the multiple layers of the nanotube [9]. HNT surface is porous with high total pore volume (TPV) and specific surface area (SSA), receptive to material modification. TPV and SSA vary among HNT, dependent on deposit origin. Lvov.Y. et al. showed HNT's nanotubular structure enables use as nanocontainers for various materials like drugs, inhibitors, vaccines, antioxidants, and enzyme immobilization supports [10].

Biopolymers such/HNT composites are utilized in diverse biological and non-biological applications, driven by environmental concerns over non-biodegradable waste and resource depletion [11]. These composites offer significantly enhanced material properties without extra processing or post-treatment. Chitosan, a biocompatible and biodegradable polysaccharide derived from chitin, is extensively utilized in tissue engineering for its ability to promote cell adhesion, proliferation, and tissue regeneration [12]. Similarly, Hydroxyapatite and pectin, offer promising potential particularly in bone tissue engineering, due to their osteoconductivity and controlled release properties. Importantly, they maintain biodegradability, leaving only inorganic minerals (clay) after degradation [13]. Polymer matrices therefore provide a versatile platform for the incorporation of these nanofillers due to their tuneable properties [14].

Poly (vinyl alcohol) (PVA) is a synthetic biomaterial used in tissue engineering and wound dressing due to its water solubility, non-toxicity, and favourable mechanical properties [15]. However, PVA is highly hydrophilic, resulting in poor cell adhesion, which can be enhanced by incorporating HNTs [16]

[17]. Polycaprolactone (PCL) is FDA-approved polyester known for its biocompatibility and slow degradation, beneficial for tissue regeneration [18]. Despite its resemblance to ECM, PCL's hydrophobicity hinders cell attachment, which can be addressed by adding clay materials and natural biopolymers to scaffolds, promoting improved cell attachment and proliferation [19]. Polylactic acid (PLA) finds widespread use in the biomedical field due to its biocompatibility and biodegradability. Its versatility and ability to degrade into harmless byproducts make PLA a promising material for various biomedical applications, including implants and surgical sutures [20].

The current thesis focuses on modifying Halloysite nanotubes (HNTs) with biopolymers chitosan and pectin and also with another mineral hydroxyapatite and reinforcing them in a polymer matrix to form nanocomposite films. The HNTs were functionalized with pectin and chitosan through the silane modification to HNTs surface. The 3-Aminopropyltriethoxysilane (APTES) was employed for the purpose. Another functionalization with nanochitosan was carried through phosphorylation. The required reaction conditions were maintained for forming the chitosan and pectin solution such as acetic acid and water respectively. Subsequently, these functionalized nanotubes were employed to fabricate nanocomposite films with varying compositions of nanofiller (1wt%, 3wt% and 5wt %) and polymer using the solution casting technique.

The fabricated nanocomposite films underwent characterization using various instrumental methods including Fourier transform infrared spectroscopy (FTIR), Powder X-ray diffraction (PXRD), thermal analysis (TGA and DSC), Universal tensile machine (UTM), Scanning Electron Microscopy (SEM), Atomic Force Microscopy (AFM), Transmission Electron Microscopy (TEM) and Water Contact Angle (WCA). The swelling behaviour of the nanocomposite films was assessed in phosphate-buffered saline at pH 7.4, and in vitro enzymatic degradation was conducted in the presence of lysozyme (egg albumen). Hemolysis studies were performed using human erythrocytes, while in vitro biocompatibility and cytocompatibility assessments, such as cell proliferation, adhesion, and migration, were carried out using mouse embryonic fibroblast (NIH3T3) cell lines. The potential applications of the nanocomposite films in biomedical fields such as tissue engineering, wound healing and bone regeneration were explored. The findings of the study are presented and discussed in seven chapters as outlined below.

Chapter 1: General Introduction

1.1 Polymer nanocomposites
1.2 Halloysite nanotube
1.3 Biopolymers and biomaterials

1.4 Polymer nanocomposites in biomedical field

1.5 References

Chapter 2: a) Literature review

b) Materials and methods

c) Characterisation techniques

Chapter 3: Functionalization of halloysite nanotube with the biopolymers such as Pectin and chitosan and with hydroxyapatite nano powder.

3.1. Functionalization of HNTs with APTES and pectin and its characterisation techniques.

3.1.1 Introduction

3.1.2 Modification route of Halloysite with silane and other chemicals.

3.1.3 Characterizations of the pectin modified HNT and its results and discussions

3.2. Functionalization of HNTs with chitosan glutaraldehyde and its characterisations.

3.2.1 Introduction

3.2.2 Modification route of Halloysite with glutaraldehyde chitosan and other chemicals.

3.2.3 Characterization techniques of the glutaraldehyde-chitosan modified HNT and its results and discussions

3.3. Functionalization of HNT with nano-chitosan and its characterisations.

3.3.1 Introduction

3.3.2 Modification process of Halloysite with phosphoric acid and synthesis of nano-chitosan inside the lumen

3.3.3 Characterization methods of the nano-chitosan modified Halloysite and its results and discussions

3.4. Functionalization of HNT with hydroxyapatite and its characterisation.

3.4.1 Introduction

3.4.2 Modification route of Halloysite with hydroxy apatite nano powder.

3.4.3 Characterizations of the hydroxy apatite modified HNT and its results and discussions

3.5. References for the modification processes involved

Chapter 4a: Preparation of Pectin modified halloysite nanotube reinforced poly (vinyl alcohol) nanocomposite films for potential tissue engineering applications.

4a.1. Preparation of pectin HNT/PVA nanocomposite films

4a.2. Characterization techniques and its results and discussions

4a.3. Enzymatic degradation and swelling study.

4a.4. Tissue engineering application studies.

Chapter 4b: Preparation of pectin modified halloysite nanotube reinforced polycaprolactone nanocomposite films for potential wound healing applications.

4b.1. Preparation of pectin HNT/PCL nanocomposite films

4b.2. Characterization techniques and its results and discussions.

4b.3. Enzymatic degradation and swelling study.

4b.4. Wound healing application studies.

Chapter 5: Preparation of chitosan-HNT (Schiff base) reinforced poly (vinyl alcohol) nanocomposite films for potential biomedical applications.

5.1. Preparation of chitosan-HNT /PVA nanocomposite films

5.2. Characterization methods

5.3. Swelling behaviour and enzymatic degradation studies.

5.4. Biomedical applications.

Chapter 6: Preparation of nanochitosan HNT reinforced polycaprolactone nanocomposite films for bone tissue engineering application.

6.1. Preparation of nanochitosanHNT/PCL nanocomposite films

6.2. Characterization methods and its results and discussions

6.3. Swelling behaviour and enzymatic degradation studies.

6.4. Bone tissue engineering application studies.

Chapter 7: Preparation of halloysite nanotube with hydroxyapatite formation and its PLA nanocomposite films for bone tissue engineering.

7.1. Preparation of hap- HNT/PLA nanocomposite films

7.2. Characterization

7.3. Swelling behaviour and enzymatic degradation studies.

7.4. Bone tissue engineering application.

Chapter.1: General Introduction

This section serves as an introduction, providing an in-depth exploration of the historical evolution and pivotal contributions of polymer nanocomposites in the field of biomaterials. It highlights the advantages of Halloysite nanotubes (HNTs) in biomedical applications, detailing their unique properties and potential. Additionally, it delves into the general processes used to modify HNTs to enhance their functionality and compatibility. The discussion extends to the integration of biopolymers, emphasizing their significance in improving material performance. Moreover, the section elaborates on the various polymer matrices employed, along with their specific roles and applications in tissue engineering.

Chapter.2: a) Literature review

This chapter presents a comprehensive review of the literature that informed the selection of materials and methodologies employed in the current research. It provides an in-depth analysis of studies on the functionalization of Halloysite nanotubes (HNTs) with pectin, chitosan, and hydroxyapatite. Additionally, the chapter explores the development of nanocomposite films using polymer matrices such as polyvinyl alcohol

(PVA), polycaprolactone (PCL), and polylactic acid (PLA), highlighting their significance and applications in the context of the research objectives.

Chapter.2: b) Materials and methods

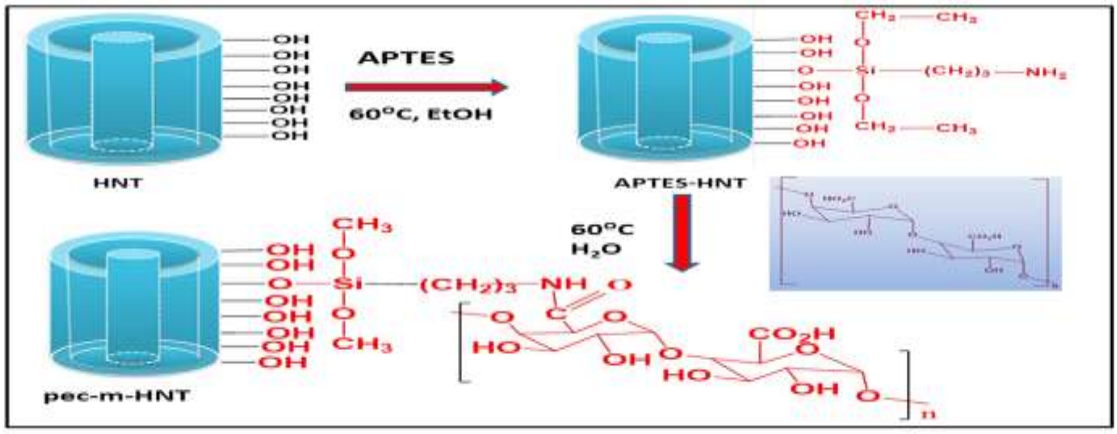
This chapter offers an in-depth account of the materials and methodologies used in the functionalization of Halloysite nanotubes (HNTs) and the fabrication of nanocomposite films. It thoroughly explains the techniques applied for characterizing both the functionalized HNTs and the resulting nanocomposite films. Detailed descriptions of the experimental procedures for assessing swelling behaviour and conducting in-vitro enzymatic degradation studies are provided. Furthermore, the chapter outlines the methods used for cellular studies, including the evaluation of cell proliferation, adhesion, and migration. In addition, it covers the experimental approaches employed to assess hemolysis and investigate in-vitro bone tissue regeneration activities, offering a holistic view of the research processes undertaken.

Chapter.2: c) Characterization techniques

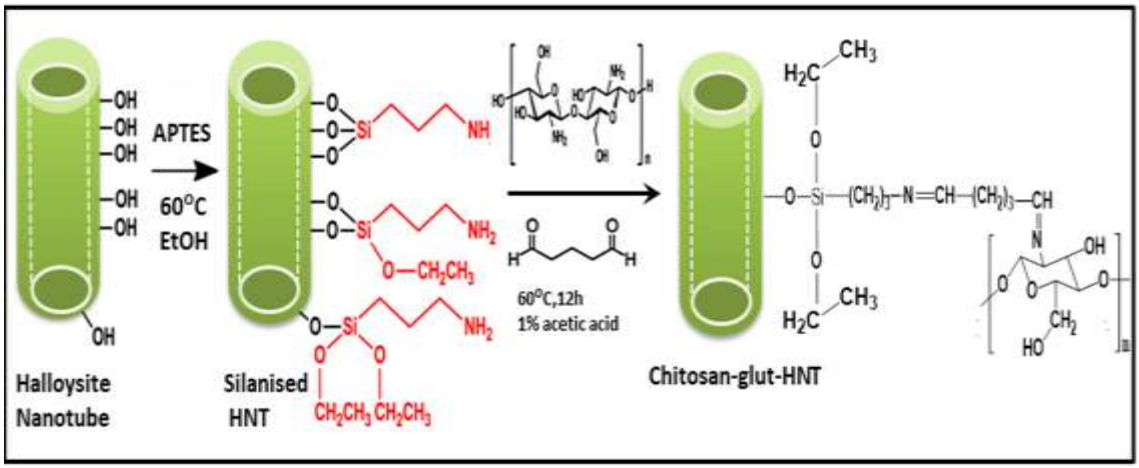
The present chapter offers a thorough overview of the various characterization techniques used to analyze Halloysite nanotubes (HNTs) modified with pectin, chitosan, and hydroxyapatite. It also details the preparation process of nanocomposite films using the solution casting method for polymers such as polyvinyl alcohol (PVA), polycaprolactone (PCL), and polylactic acid (PLA). Furthermore, the chapter explains the corresponding characterization methods used to evaluate the properties and performance of these nanocomposite films, providing a comprehensive understanding of the analytical approaches involved.

Chapter.3: Functionalization processes for the HNT

It explains the various steps involved in the modification process of the pristine halloysite nanotubes with pectin, chitosan, nanochitosan and the mineral hydroxyapatite. The characterization techniques such as FTIR, PXRD, TGA-DSC, FESEM and TEM are clearly explained.



Scheme.1 Functionalization of halloysite nanotube with pectin.



Scheme.2 Functionalization of halloysite nanotube with chitosan.

Chapter 4a: Functionalization of halloysite nanotube with pectin and its poly (vinyl alcohol) nanocomposite films for potential tissue engineering applications.

This chapter deals with the synthesis of PVA nanocomposites reinforced with the pectin modified HNTs into the matrix. The film forming solution was made using distilled water and the well-known solution casting technique. The as obtained nanocomposites films of varying weight percentages of the nanofillers are well characterised using FTIR, PXRD, DSC-TGA, FESEM and AFM. The test samples were also tested for mechanical and water contact angle measurements. The biocompatibility of the films has been tested using the hemolysis, enzymatic degradation, cell adhesion and proliferation assays are also explained in the chapter. Using the results the tissue engineering application of the films is established.

Chapter 4b: Functionalization of halloysite nanotube with pectin and its polycaprolactone nanocomposite films for potential wound healing applications.

This particular chapter brings out the synthesis of PCL nanocomposites reinforced with the pectin modified HNTs into the matrix. The films were made using distilled water and the well-known solution casting technique. The nanocomposites films of 1, 3, and 5wt% of nanofillers are well analysed using FTIR, PXRD, DSC-TGA, FESEM and AFM. The film samples were also analysed for mechanical behaviour and water contact angle studies. The biocompatibility of the films has been tested using the haemolytic and enzymatic degradation. The cell adhesion and proliferation assays along with the wound healing assays were performed on the mouse fibroblasts. The data have added up positive results to the biocompatible nature of the polymer nanocomposite films.

Chapter 5: Functionalization of halloysite nanotube with chitosan forming Schiff base and its poly (vinyl alcohol) nanocomposite films for potential biomedical healing applications.

This chapter describes the fabrication of PVA nanocomposite films and its characterisations using FTIR, PXRD, DSC-TGA, FESEM and AFM techniques. The evaluation of the mechanical behaviours and hydrophilicity/hydrophobicity is done using the UTM and WCA analytical methods. The cytocompatibility studies and the cell proliferation-adhesion are done using the NIH3T3 fibroblast cell lines. The results obtained for the *in-vitro* cellular studies show that the synthesised films are susceptible towards biomedical applications.

Chapter 6: Functionalization of halloysite nanotube with nanochitosan formation and its PCL nanocomposite films for bone tissue engineering.

The chapter describes the synthesis of the polycaprolactone films containing the nanochitosan embedded halloysite nanotubes reinforced in the matrix. The prepared polymer nanocomposite films were well characterised by FTIR, PXRD, DSC-TGA, FESEM, AFM and WCA methods. The mechanical and wettability of the study were also analysed accordingly. The enzymatic degradation and the hem compatibility assay are performed in PBS at physiological pH. The cell adhesion, cell proliferation and alkaline phosphatase assay for the bone tissue regeneration studies. The *in-vitro* studies were performed using the NIH3T3 mouse fibroblasts. All the results of the biomedical studies have proved the applicability of the nchito-HNT/PCL nanocomposites in bone tissue engineering.

Chapter 7: Functionalization of halloysite nanotube with hydroxyapatite formation and its PLA nanocomposite films for bone tissue engineering.

This particular chapter reveals the fabrication of PLA films reinforced with the hydroxyapatite modified HNT. After the reinforcement of the filler into the matrix, the film samples are characterised with FTIR, PXRD, DSC-TGA, FESEM, AFM and water contact angle measurements. The haemolytic assay emphasising the blood compatibility of the films is done in the phosphate buffer solution (PBS) at pH of 7.4 and the results for all the samples showed less than 5% of hemolysis. The in-vitro enzymatic degradation is performed by taking egg albumin as the enzyme. The swelling capabilities of the nanocomposites were analysed using PBS gain. The cytotoxicity was established with

Summery and conclusions

The studies referenced in the preceding sections have been thoroughly reviewed, and no significant similarities have been identified in the literature. Therefore, the conclusions drawn in this work are presented in the communicated manuscripts. The data collected highlights the promising potential applications of the nanocomposite films, particularly after reinforcing the modified Halloysite nanotubes (HNTs), for various biomedical applications. These findings underscore the advancements in the field and the value of these nanocomposite films in addressing biomedical challenges.

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List of publications

1. Shabeena, M., Kouser, S., Prabhu, A., Nagaraja, G. K., Warale, D., & Manasa, D. J. (2023). Biocompatible pectin-functionalised-halloysite loaded poly (vinyl alcohol) nanocomposite films for tissue engineering applications. *Journal of Drug Delivery Science and Technology*, *82*, 104320.

2. Shabeena, M., S Warale, D., Prabhu, A., Kouser, & Manasa, D. J. Nagaraja, G. K., (2023). Pectin wrapped halloysite nanotube reinforced Polycaprolactone films for potential wound healing applications. *International Journal of Biological Macromolecules*, 130140.

3. Shabeena, M., Kouser, S., Prabhu, A., Warale, D., Manasa, D. J., & Nagaraja, G. K. (2024). Unravelling the tissue regenerative nature of marine polysaccharide chitosan embedded halloysite reinforced poly (vinyl alcohol) nanocomposite films. Discover Polymers, 1(1), 3.

List of publications under communications

1. Shabeena, M., Prabhu, A., & Manasa, D. J. Nagaraja, G. K., Phosphorylated halloysite leading to the synthesis of nano-chitosan and its polycaprolactone films- a galore of phosphorous for bone tissue engineering (**under review**).
2. Shabeena, M., S Warale, D., Prabhu, A., Kouser, & Manasa, D. J. Nagaraja, G. K., Hydroxyaptite functionalized halloysite reinforced PLA nanocomposites for bone tissue engineering applications.

List of national/international conferences attended

1. **Shabeena M**, Nagaraja G. K., Poster presentation entitled "The study on budding utility of pectin grafted halloysite and its poly (vinyl alcohol) nanocomposite films at International Conference on Fundamental and Applied Sciences (ICFAS 2021) organized by Faculty of Science and IQAC, Hazarimal Somani College of Arts and Science, Shri Manubhai Maneklal Sheth Junior College of Arts during 24-26th March, 2021.
2. **Shabeena M**, Nagaraja G. K., Poster presentation entitled "Pectin modified halloysite nanotube containing PVA nanocomposite films for biomedical applications" at the International Conference on Frontier Reseach in Chemical Sciences aorganised by Jyothi Nivas College, Bhandra, during 11-13th November, 2021.
3. **Shabeena M**, Nagaraja G. K., Poster presentation entitled "Pectin wrapped halloysite nanotube reinforced Polycaprolactone films for potential wound healing application in the International Conference on Condensed Matter Physics and Applications organized by the Department of Physics, MAHE, Manipal on 14th and 15th of December, 2023.
4. **Shabeena M**, Nagaraja G. K., Oral presentation on " Nanochitosan embedded HNT and its polycaprolactone films for bone tissue engineering" at the International Conference on Transformative Chemistry for a Sustainable Future organised by St. Aloysius (Deemed to be University) on 15th March 2024. **The presentation was awarded with the Best Paper Award**.

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